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Protein kinase C (PKC) is a key signal transduction pathway for regulating cell growth and differentiation. Perturbations in the PKC signaling pathway are widely recognized to be important in carcinogenesis since PKCs are the major cellular receptors for tumor promoting phorbol esters. PKC is actually a family of distinct isozymes that catalyze phospholipid-dependent protein phosphorylation. The role of individual isozymes in cellular processes has not yet been defined. The goal of this proposal is to study the role of individual PKCs in regulating mammary tumor growth and metastatic conversion.

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INTRODUCTION:

Changes in cellular and molecular functions that contribute to the development and progression of breast cancer are largely undefined. Changes in activities and/or expression levels of components of growth regulatory signal transduction pathways that correlate with tumor progression have been noted. Understanding the functional significance of these biochemical correlates holds the promise for developing new and alternative strategies for prevention, detection, diagnosis and treatment of breast cancer. Protein kinase C (PKC) is a family of ubiquitously expressed enzymes known to be important in regulating basic mechanisms of cell growth and differentiation. PKC is also considered to be a significant signaling pathway in tumor promotion/progression since PKCs are the major cellular receptors for tumor promoting phorbol esters. **The purpose of this grant was to explore the role of PKC isozymes and substrates in the transition of a normal breast cell into a cell with altered growth potential and subsequently into a cell with invasive, metastatic potential.**

PKC and Mammary Cell Biology. Epidemiological studies have not yet identified a dominant etiology for breast cancer. However, since many of the major risk factors (age at menarche, age at first full-term pregnancy, age at menopause) are linked to natural hormone production, it is likely that endogenous hormone levels play an important role in mammary tumor promotion and progression. There is evidence that PKC is involved in two important aspects of mammary carcinogenesis: estrogen-independence and proliferation [reviewed in (1)].

- *PKC and Estrogens.* In MCF-7 cells, PKC activation was associated with decreased estrogen signaling. Phorbol esters caused down regulation of estrogen receptor message and modulation of AP-1/estrogen receptor interactions (2-4). Overexpression of PKCα in MCF-7 cells decreased estrogen receptor mRNA and estrogen-dependent gene expression (5). PKC has been implicated in the loss of hormonal dependence that accompanies mammary tumor progression since PKC expression was increased in estrogen-independent compared to estrogen-dependent human breast cancer cells (6). Epidemiological studies have indicated a correlation between estrogen-independence and up regulation of EGF signaling pathway components, although a direct role for EGF signaling in developing estrogen-independence has not yet been demonstrated. Since EGF stimulates PKC activity in a variety of cells, PKC is also implicated in regulation of estrogen sensitivity.
- *PKCs and Proliferation.* Several investigators have studied the effects of phorbol esters on growth and differentiation of cultured mammary cell lines. In summary, the reported phorbol ester effects are variable and may depend on individual properties of different cell lines. It is important to note that phorbol esters stimulated proliferation, inhibited differentiation and altered morphological development of undifferentiated, primary cells (7). Since this is the cell population most sensitive to chemically induced mammary carcinogenesis, these results indicate a role for PKC in early events in mammary cell transformation. PKC-dependent effects are likely to be mediated through

a variety of physiological regulators of mammary cell growth and differentiation which have been shown to work at least partially through PKC-dependent pathways:

•*PKC and prolactin.* Prolactin-stimulated growth of cultured mouse mammary epithelial cells is accompanied by increased PKC activity and redistribution of PKCs from soluble to particulate fractions (8).

•*PKC and EGF.* EGF is among the most potent of growth factors for cultured mammary epithelial cells (9). EGF stimulates the activity of PLCγ to produce DAG, the endogenous regulator of PKC activity. A correlation between increased EGF receptor and PLCγ levels in estrogen receptor negative primary human breast cancers has been noted (10), and may suggest that upregulation of this pathway contributes to estrogen-independence of human tumors.

•*PKC and TGF*β. TGFβ is important for ductal development and differentiation (11). Recent evidence indicates that phosphatidylcholine-specific phospholipase C and PKC may be early intermediates in TGFβ signaling pathways that lead to activation of gene transcription (12).

Taken together, these results indicate an important role for PKC as a downstream regulator and/or effector of mammary growth and differentiation. The overall goal of this IDEA grant was to define the role(s) of PKC signaling pathways in the loss of growth control that accompanies the conversion of preneoplastic to neoplastic lesions and development of metastatic potential in mammary epithelial cells.

Our hypothesis is that PKC isozymes are important for several aspects of mammary tumor promotion and progression including increased growth potential, tumor incidence and invasiveness. Two experimental rodent models were used to study PKC signaling in mammary carcinogenesis. The first (in collaboration with D. Medina) emphasizes early events in transformation from preneoplastic to neoplastic cells. The second (in collaboration with D. Welch) is used to identify PKC signaling events associated with metastatic potential. We have demonstrated changes in levels of individual PKCs and PKC substrates in tumors as compared to preneoplasias and in highly metastatic cells compared to less metastatic cells. **In future studies, we will test the hypothesis that the observed changes in levels of PKC signaling components drive the transformation and are not merely correlative.** To do this we have manipulated the activity levels of individual PKCs in recipient cells by expressing active PKCs or dominant negative PKC constructs, and monitored effects on *in vitro* and *in vivo* growth properties. Linking increased activity of an individual PKC to increased mammary cell growth potential, decreased tumor incidence or increased metastatic potential would identify a new therapeutic target for limiting mammary carcinogenesis.

Our preliminary data also indicate that expression of certain PKC substrates is down modulated with progressive transformation. To test the hypothesis that loss of these substrates contributes to loss of growth control, we propose to reintroduce expression of these substrates and determine the effects on cell growth in culture and *in vivo*. These studies will determine if individual PKCs or substrates are suitable targets for developing breast cancer treatment therapies and/or useful markers for diagnosis and prognosis.

BODY:

Mammary Tumor Promotion/Progression. **A major goal of this project was to identify biochemical correlates of the transition from preneoplastic to neoplastic growth potential with regard to PKC signaling pathway components.** As with other tissues, the development of mouse mammary tumors occurs through definable stages. The earliest events are associated with a mutation (either inherited or due to unknown exogenous influences) to produce an "initiated" cell with altered growth potential. Additional genetic and epigenetic events produce cells that are increasingly abnormal. The principle limitation of PKC studies in breast has been the limited focus on transformed epithelial models in culture. Such models are not able to consider the impact of stromal-epithelial interactions on growth regulation. The stage-wise progression of neoplastic transformation has been recapitulated in an *in vitro-in vivo* model system of mouse mammary carcinogenesis developed by Dan Medina (Baylor) and has been used to study and identify biochemical correlates of mammary tumor development. Properties of normal, preneoplastic and neoplastic mouse cell populations have been described (13-15). To establish this model, cell lines were generated from primary mouse mammary epithelial cells expanded in three-dimensional collagen gel culture and then passaged into cleared fat pads. Certain of these preneoplastic cell lines can be transplanted indefinitely (ex vivo survival for several generations) without producing preneoplastic lesions. This distinguishes them from normal cells, and they have been named extended life (EL) lines. Other lines of transformed mammary (TM) cells develop preneoplastic lesions and tumors *in vivo* at characteristic rates that reflect their stage of transformation. Cell lines in the Hyperplasia I group do not produce tumors within the first 15 transplantation generations. In contrast, cell lines in the Hyperplasia II and III groups produce tumors at earlier transplantation generations and with increased incidence. Comparisons of the *in vitro* and *in vivo* properties of the several TM cell lines was used to generate a model for mammary tumor progression.

Normal → Immortalized *(EL11, EL12)*→ Hyperplasia I *(TM3, TM2L)* → Hyperplasia II *(TM10, TM12, TM40)* → Hyperplasia III *(TM2H, TM4)* → Metastases *(TM40)*

These lines have been useful for identifying the nature and function of molecular changes associated with mammary carcinogenesis (15,16). Using this model, we have identified reproducible differences in levels of certain PKCs and substrates between hyperplastic lesions and tumors (see below). The combination of the *in vitro-in vivo* approaches provides flexibility in designing mechanistic studies to determine the functional role of identified biochemical changes in tumor development. **To determine if the observed biochemical changes are functional correlates of mammary tumor progression, we will extend these studies and prepare cell populations with increased expression of individual PKCs or PKC dominant negative constructs and assess effects of introduction of these genes on growth and tumor incidence *in vivo*.**

PKC Isozymes in Preneoplastic and Tumor Tissues. As part of studies initiated under my IDEA grant, in collaboration with D. Medina (Baylor), we compared levels of PKC isozymes and substrates in non-tumorigenic hyperplasias, tumorigenic hyperplasias and neoplasias derived from these cell lines. Three types of transformed mammary cell lines were used in these studies: alveolar hyperplasia lines that characteristically produce tumors at a low incidence or a relatively high incidence (TM12 or TM4). TM40 produces relatively few tumors; however, many of these tumors are metastatic.

Levels of PKC isozymes in the preneoplasias **(PrN)** and tumors **(T)** were compared with levels in pregnant glands, which have a comparable level of mitogenic activity. PKC levels were determined by immunoblots stained with PKC isozyme-specific antibodies and quantitated by densitometry **(Fig. 1 and Table 1)**. The results demonstrate that PKC isozyme contents in pregnant tissue and preneoplastic outgrowths are comparable. However, paired comparisons of levels in tumor tissues to the corresponding preneoplasias revealed significant and reproducible differences. Compared to preneoplastic and pregnant tissue, tumors produced from all five of the TM lines analyzed had increased levels of PKCδ and ζ and decreased levels of PKCε. PKCα levels were not reproducibly changed. Since the pregnant gland, the preneoplasias and tumors are all comprised of proliferating cell populations, it seems likely that the quantitative changes in PKCs observed in tumor tissues are not simply related to differential proliferative states. In fact, levels of each of the isozymes in hyperplasias and pregnant glands were not significantly different (data not shown). Furthermore, since increased proliferation has been associated with down modulation of PKC proteins, it is unlikely that the increase in proliferation in the tumors can account for increases in PKCs δ and ζ. **These preliminary results suggest the hypothesis that increased expression of individual PKCs can influence the tumorigenic potential of transformed mammary epithelial cells.**

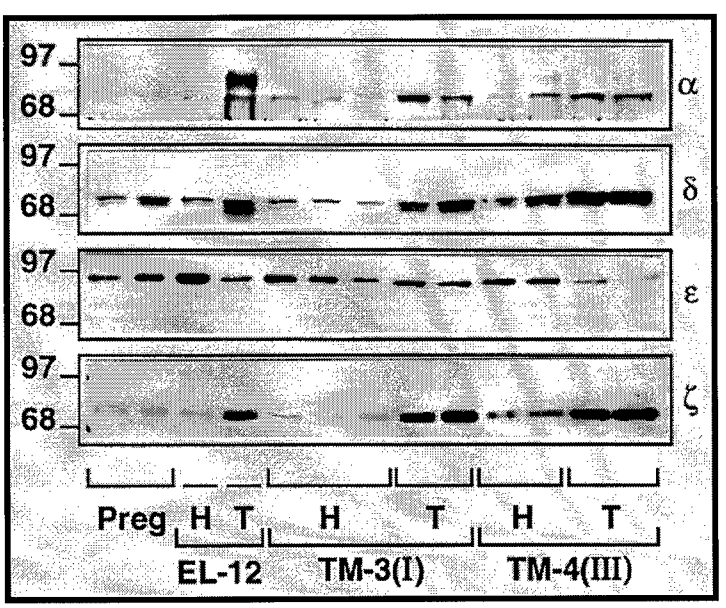


Fig. 1: *PKC isozyme profiles in preneoplastic and neoplastic mouse mammary tissues.* Tissue homogenates were prepared from mammary tissues frozen in liquid nitrogen. Aliquots (50 μg) were separated by SDS-PAGE and blotted to nitrocellulose. Blots were probed with affinity-purified PKC isozyme-specific antibodies. Fig. 1 shows representative samples. Table 1 summarizes data from several experiments in which isozyme levels were quantitated by densitometry.

Table 1. PKC Isozymes in Preneoplastic and Tumor Tissues.

Lesion Type	Cell Line	PKCα, T/H*	PKCδ, T/H*	PKCε, T/H*	PKCζ, T/H*
Hyperplasia I	TM3	2.7	3.1	0.9	9.5
	TM2L	0.7	1.3	0.4	2.1
Hyperplasia II	TM12	0.5	1.9	0.2	2.3
Hyperplasia III	TM4	2.8	2.1	0.3	3.1
Hyperplasia II/ Metastases	TM40A	1.4	2.4	0.3	7.7
Mean ± S.D.		1.3±1.1	2.2±0.7	0.42±0.3	4.9±3.4

*** H, hyperplasia; T, tumor**

Mammary Metastatic Progression. Tumor metastasis is the most compelling problem in cancer treatment. Primary tumors can often be effectively treated with surgery and radiation; however, metastatic cells that have colonized other tissues pose an additional threat. In order to spread from the primary sites, malignant cells must invade surrounding normal tissues, penetrate blood and/or lymphatic vessels, dislodge from the primary tumor mass, disseminate to distant sites, arrest in the lymph nodes or microvasculature of distant organs, and finally invade the parenchyma and stroma at these sites to survive and grow into a metastatic colony. It is clear that metastasis is a complex process requiring many biochemical alterations for productive tumor spread. To identify biochemical correlates of the PKC signaling pathway associated with metastatic potential, we have studied PKCs and PKC substrates in clonal cell lines derived from the 13762 rat mammary primary adenocarcinoma or lung metastases (17,18). Clones derived from the primary tumor have low **(L)** or moderate **(M)** metastatic potential (MTC and MTF7, respectively) in the *in vivo* metastasis assay. The highly **(H)** metastatic cells used in these studies were cloned from lung metastatic colonies (MTLn3).

Due to selection during the sequential events required for metastatic growth, the genetic composition of secondary tumors differs from that of primary tumors. Thus, comparison of primary to secondary growths provides an opportunity for defining genetic alterations that are important for metastatic growth. The 13762 rat mammary adenocarcinoma system has been used to isolate metastasis-associated genes by screening subtraction libraries prepared from low and high metastatic clones (19). One of these genes, *mta1*, is a novel sequence that is upregulated in MTLn3 cells and may play a role in breast cancer invasion and metastasis (20). MTLn3 cells also express high levels of the EGF receptor compared to MTC cells (21). Functional significance of this biochemical change was demonstrated by introducing EGF receptor expression into the MTC cells. Increased expression of EGF receptors caused an EGF-dependent

increase in adherence to extracellular matrix components *in vitro* and in lung metastases *in vivo*. Thus, expression and ligand-dependent stimulation of EGF receptors appears to be at least in part responsible for the differential metastatic potential of the MTC and MTLn3 cells (21). These results are of potential significance to human breast cancer since there is a negative association between EGF receptor levels and relapse free survival, a positive association between EGF receptor levels and loss of endocrine sensitivity and an increase in EGF receptor levels in breast tumor metastases compared to the primary tumor [summarized in (21)].**We have identified changes in levels of PKC isozymes that may also contribute to the increased metastatic potential of the MTLn3 cells (see below). Future experiments will address the functional significance of these biochemical changes.**

PKC Isozymes in Progressively Metastatic Mammary Epithelial Cells. We have compared PKC isozyme contents in MTC, MTF7 and MTLn3 cells (L, M, H, respectively) in collaboration with D. Welch (Hershey) . PKC levels were compared in high density cultures to minimize potential differences associated with different proliferation rates. Under these conditions, the PKC isozyme profiles of the low and moderate metastatic cells were comparable. Thus, two independent clones isolated from the primary tumor had similar PKC isozyme profiles. Compared to these cells, PKCδ and ζ levels were increased (1.8 and 2-fold, respectively) in the highly metastatic cells, whereas PKCε levels were decreased. In contrast, PKCα levels did not change **(Fig. 2)**. Differences in protein content were directly related to differences in message levels measured in ribonuclease protection assays **(Table 2)**. The differences in PKC levels could not readily be attributed to differences in proliferation rates since qualitatively similar results were observed in more rapidly growing, low density cultures. Since the MTLn3 cells were selected *in vivo* for their metastatic potential, it is likely that they have acquired genetic changes relevant for metastatic growth. **The observed biochemical differences in PKC levels suggest the hypothesis that specific changes in PKC isozyme contents may be related to the increased metastatic potential of mammary epithelial cells.** To test this hypothesis, we propose to increase the expression of individual PKCs in the MTC cells and introduce RD dominant negative constructs in MTF7 lines and evaluate how these interventions influence biochemical correlates of metastases in culture and metastatic potential *in vivo*.

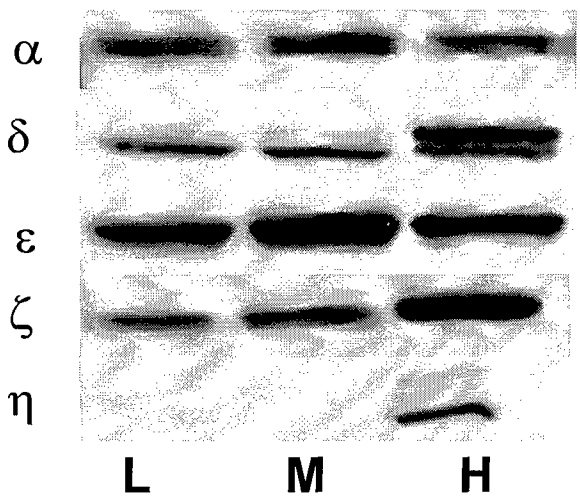


Fig. 2: *PKC isozyme profiles in mammary epithelial cells with low (L), moderate (M) or high (H) metastatic potential.* Aliquots of cell extracts (25 μg) were separated by SDS-PAGE and blotted to nitrocellulose. Blots were stained with isozyme-specific antibodies.

Table 2. PKC Isozyme Message Levels in Mammary Epithelial Cell Lines

Cell Line		PKCα	PKCδ	PKCε	PKCζ
MTC	(L)	[1]0.72±0.36	2.1±0.32	9.2±1.5	[2]N.D.
MTF7	(M)	0.69±0.09	2.5±0.81	7.1±1.3	N.D.
MTLn3	(H)	0.70±0.21	4.8±0.84	4.6±1.5	N.D.
H/L		1.0	2.3	0.5	N.D.

[1]Mean ± standard deviation calculated from densitometry values derived from three experiments using three different RNA preparations.
[2]N.D. = Not Detectable.
Total RNA was collected from the low (L), moderate (M) or highly (H) metastatic mammary cells. Message levels for individual PKCs were determined by ribonuclease protection assays using isozyme selective probes. Results were quantitated by densitometry and summarized in this table.

PKC Substrates in Mammary Cells and Tissues. We have cloned several PKC substrates according to their high affinity for PKCs in an *in vitro* binding assay (22). Affinity-purified antibodies to the expressed sequences were used to stain immunoblots to compare expression of PKC substrates during mammary carcinogenesis. Levels of two novel sequences, clones 34 and 72, were markedly reduced in tumor *vs.* preneoplastic tissues and in highly metastatic *vs.* less metastatic cultured cells. In contrast, levels of the PKA binding protein AKAP95 increased **(Fig. 3A)**. Levels of other PKC binding proteins did not change. Changes in protein levels correlated with changes in message levels as determined in Northern blots **(Fig. 3B)**. Loss of PKC binding proteins/substrates could cause disruption of PKC signaling pathways due to loss of targeting of PKCs and/or due to loss of the functions of these proteins themselves. In future experiments, we will reintroduce expression of these binding proteins in the cells and monitor effects on growth and metastasis.

Role of PKCδ in Metastatic Progression. The increased PKCδ content of the highly metastatic cells suggested that this isozyme may potentially play a role in metastatic progression. To establish a functional correlate, we developed cell lines that conditionally express a dominant negative construct of PKCδ. The construct contains the N-terminal regulatory domain of PKCδ which includes the autoinhibitory pseudosubstrate motif, the phorbol ester binding sites, phospholipid recognition domains and does not have any catalytic activity. The inhibitory constructs were placed behind a sheep metallothionein promoter to permit conditional expression of this potentially growth inhibitory sequence. As shown in **Fig. 4**, expression of RDδ was tightly regulated in the moderately metastatic MTF7 cells. Expression of RDδ did not influence the localization or levels of endogenous PKCδ **(Fig. 4)** or other isozymes in

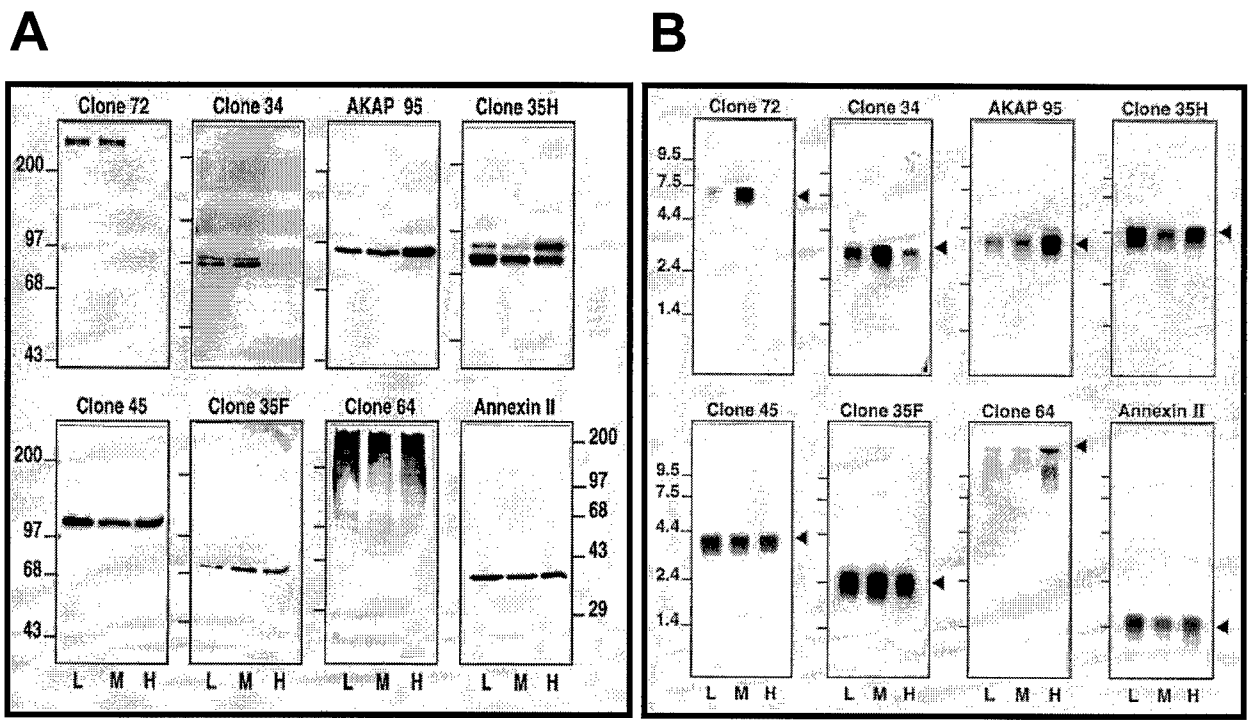


Fig. 3: *Levels of PKC substrates/binding proteins in mammary adenocarcinoma cells.* **(A)** Whole cell lysates prepared from cultures of low (L), moderate (M), and high (H) metastatic potential were loaded onto 7.5% or 10% gels (50 μg/lane). Cell proteins were separated by SDS-PAGE, blotted to nitrocellulose and probed with affinity-purified antibodies directed against a variety of PKC substrates and an A-kinase anchoring protein, AKAP 95. **(B)** PolyA$^+$ mRNAs isolated from confluent cell cultures of low (L), moderate (M) and high (H) metastatic potential were loaded (2.5 μg/lane) and separated on a 1% agarose-formaldehyde gel. RNA was blotted to Hybond nylon membrane and blots were hybridized with ^{32}P-labeled cDNA probes to the binding proteins as indicated.

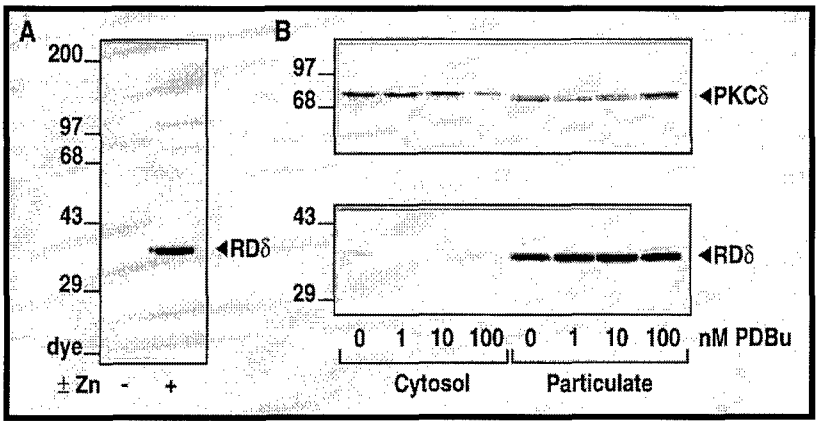


Fig. 4: *Conditional expression of RDδ in moderately metastatic MTF7 cells.* MTF7 cell clones transfected with pMTH3 RDδ expression vector were selected by growth in G418 containing medium. Cell lysates were prepared from cells grown in the presence and absence of Zn (100 μM). **A.** Aliquots of lysates were separated by SDS-PAGE, blotted to nitrocellulose and stained with antibody directed towards the N-terminal fusion protein sequence of the RDδ construct. **B.** Cell cultures were treated with Zn (100 μM) for 24 h to induce RDδ expression. Cells were then treated with indicated concentrations of PDBu for 10 min. Cell lysates were fractionated by centrifugation (100,000 xg). Levels of endogenous PKCδ and RDδ in cytosol and particulate fractions were determined on immunoblots stained with antibodies directed towards the C-terminus of PKCδ or the N-terminal fusion protein sequence of the RDδ construct.

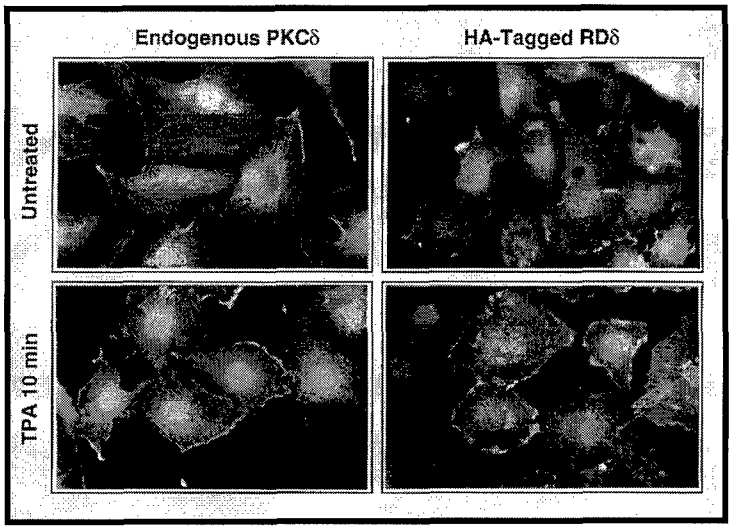


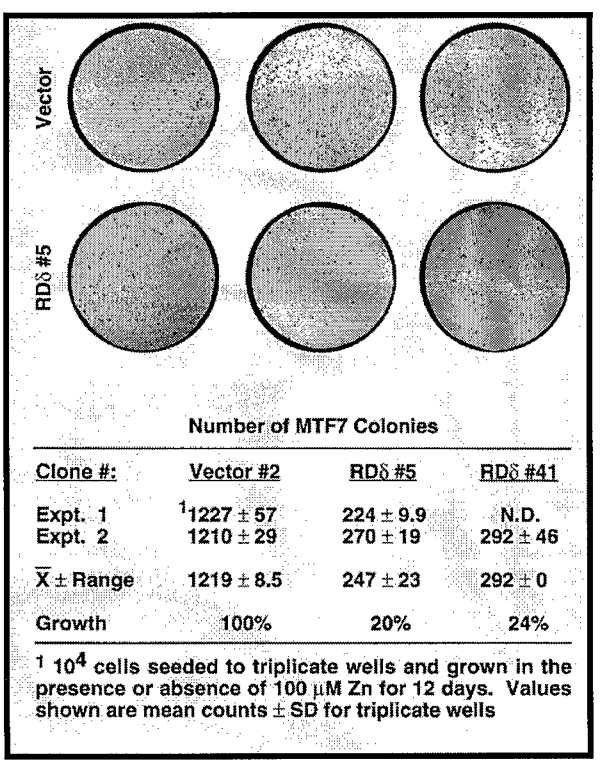
Fig. 5: *Localization of endogenous PKCδ and ectopic RDδ in MTC rat mammary adenocarcinoma cells.* **(Left)** MTC cells were fixed and stained with affinity-purified δ14K antibody (3 μg/ml). In resting cells, PKCδ is diffusely localized throughout the cytoplasm. Activation with phorbol esters (TPA, 10 min) induces redistribution to the plasma membrane.
(Right) MTC cells expressing the HA-tagged RDδ construct under the control of the sheep metallothionein promoter were fixed and stained with a commercially available monoclonal antibody to the HA tag sequence. In the absence of added zinc, expression of HA-RDδ was not detectable. After 24 hours in 100 μM zinc, HA-RDδ was detected throughout the cytoplasm. Activation with phorbol esters caused redistribution to the plasma membrane.

these cells (data not shown). Both endogenous PKCδ and exogenous RDδ were localized to cell borders and ruffles, indicating that RDδ is appropriately located to interfere with endogenous PKCδ signaling events **(Fig. 5)**. Although zinc-induced expression of RDδ had no apparent effect on anchorage-dependent growth **(Table 3)**, expression did inhibit anchorage-independent growth **(Fig. 6)**. Thus, RDδ may selectively interfere with processes involved with non-adherent cell growth.

Table 3. Effect of RDδ expression on adherent cell growth.

Clone #	-Zn	+Zn
Vector #2	3.1±0.85	3.2±1.00
RDδ #5	3.6±0.54	3.4±0.86
RDδ #41	4.4±0.66	4.4±0.31

Clones of MTF7 cells transfected with empty vector (vector #2) or the regulatory domain of PKCδ (RDδ #5 and RDδ #41) were plated ($3x10^3$ cells) in 12-well trays. Cell numbers from triplicate wells were determined after four days in culture.



Number of MTF7 Colonies

Clone #:	Vector #2	RDδ #5	RDδ #41
Expt. 1	[1]1227 ± 57	224 ± 9.9	N.D.
Expt. 2	1210 ± 29	270 ± 19	292 ± 46
$\bar{X}$ ± Range	1219 ± 8.5	247 ± 23	292 ± 0
Growth	100%	20%	24%

[1] 10^4 cells seeded to triplicate wells and grown in the presence or absence of 100 μM Zn for 12 days. Values shown are mean counts ± SD for triplicate wells

Fig. 6: *Effect of RDδ expression on non-adherent cell growth.* Growth in soft agar of the moderately metastatic MTF7 clones transfected with empty vector (vector #2) or RDδ (RDδ #5 and RDδ #41) was monitored.

Although the zinc regulated system provided tightly controlled expression, the high levels of zinc required for induction indicated that experimental metastasis assays in animals would be difficult. We therefore subcloned the epitope-tagged RDδ and wild type PKCδ constructs into a vector containing a tetracycline responsive promoter. This plasmid was cotransfected into the highly metastatic MTLn3 cells with a vector containing a neomycin resistance marker and a mutated tetracycline repressor. The mutated repressor induces (rather than inhibits) expression from the tetracycline responsive promoter [the reverse tet system, (23)]. Among the several G418-resistant clones screened, three showed very low RDδ expression in the absence of the tetracycline analogue doxycycline and high expression in the presence (data not shown). Doxycycline-induced expression of RDδ inhibited growth on plastic **(Table 4)** and in soft agar **(Table 5)**. In contrast, in similar experiments with cells constructed to conditionally express RDα, no effect on adherent or non-adherent growth was observed **(Tables 4 and 5)**. The differential effects of RDα and RDδ expression on growth inhibition indicate that inhibitor effects are specific for the PKCδ signaling pathway.

CONCLUSIONS:

Using two different rodent models for mammary tumor progression, we have found that PKCδ and ζ increase and PKCε decreases as cells become increasingly transformed. In addition, we have found that levels of two novel PKC substrates, clones 34 and 72, decrease with increasing transformation, whereas levels of other PKC substrates do not change. These results strongly suggest the hypothesis that distinct changes in PKCs and PKC substrates are common events in mammary tumor progression and that these changes may be causal rather than correlative. We have begun to test the functional significance of the observed changes by manipulating the levels of PKCs and substrates in cultured mammary cells and studying the effects of altered PKC and PKC substrate content on mammary cell growth and tumorigenesis *in vivo.* The decreased growth potential of the cells expressing dominant negative constructs of PKCδ and the corresponding increased growth potential of cells overexpressing an active form of PKCδ indicate that PKC isozymes play an important, modulatory role in mammary cell growth and metastatic potential.

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Table 4. Effect of RDδ and RDα on Adherent Cell Growth

Construct	Clone	cell number x10^4, +dox:-dox
Vector only	4	10.4/10.3
RDδ clones	δ 2/21	6.8/12.4
	δ 2/36	7.2/15.1
	δ 2/51	6.5/16
RDα clones	B61/63	28/26
	B61/2	25/26
	B61/72	18/17

Clones of the highly transformed MTLn3 cells were selected after transfections with empty vector (vector #4), RDδ (clones 2/21, 2/36 and 2/51) or RDα (clones B61/63, B61/2 and B61/72). RDδ and RDα expression were induced with doxycycline (dox) treatment. Data are reported as the ratio of colony number in the presence and absence of doxycyclin (+dox:-dox).

Table 5. Effect of RDδ and RDα on Growth in Soft Agar

Construct	Clone	Number of foci, +dox:-dox
Vector only	4	1.0±0.1
RDδ clones	2/21	0.5±0.2
	2/36	0.7±0.2
	2/51	0.4±0.3
RDα clones	B34/60	1.0±0.1
	B61/63	1.0±0.1
	B61/2	1.0±0.1
	B61/72	1.0±0.1

Clones of the highly transformed MTLn3 cells were selected after transfections with empty vector (vector #4), RDδ (clones 2/21, 2/36 and 2/51) or RDα (clones B34/60, B61/63, B61/2 and B61/72). RDδ and RDα expression were induced with doxycycline (dox) treatment. Data are reported as the ratio of colony number in the presence and absence of doxycyclin (+dox:-dox).

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